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THE DETECTION OF HEMORRHAGIC PROTEINS IN SNAKE VENOMS USING MONOCLONAL ANTIBODIES AGAINST VIRGINIA OPOSSUM (*DIDELPHIS VIRGINIANA*) SERUM

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E. E. Sánchez, C. García, J. C. Pérez and S. J. De La Zerda. The detection of hemorrhagic proteins in snake venoms using monoclonal antibodies against Virginia opossum (*Didelphis virginiana*) serum. *Toxicon* **36**, 1451–1459, 1998.—Most snakes and a few warm-blooded animals have a resistance to snake venoms because of naturally occuring antihemorrhagins found in their sera. The antihemorrhagins in serum of Virginia opossum (*Didelphis virginiana*) neutralize hemorrhagic activity by binding to hemorrhagins in snake venoms. The binding characteristic of antihemorrhagins in *D. virginiana* serum was used to develop a five-step western blot. The detection of hemorrhagic proteins were measured indirectly with antihemorrhagins in Virginia opossum serum and with DV-2LD#2, a monoclonal antibody specific for Virginia opossum antihemorrhagins. Snake venoms were separated by native-PAGE, transferred to a Millipore Immobilon®-P membrane and then incubated with crude Virginia opossum serum. The hemorrhagins in snake venom bind to antihemorrhagins in Virginia opossum serum which react with DV-2LD#2 a monoclonal antibody that is specific for Virginia opossum antihemorrhagins. DV-2LD#2 monoclonal antibody inhibits antihemorrhagic activity in Virginia opossum serum when mixed in equal amounts. The inhibition of antihemorrhagins by DV-2LD#2 monoclonal antibody suggests specificity. DV-2LD#2 monoclonal antibody does not recognize antihemorrhagins in gray woodrat (*Neotoma micropus*) serum. The five-step western blot reveals two well-defined bands which represent hemorrhagins found in Western diamondback rattlesnake (*Crotalus atrox*) venom. Venoms from 15 different snake species were examined to determine the usefulness of the five-step western blot. Other hemorrhagic venoms (Great Basin rattlesnake (*C. viridis lutosus*), Prairie rattlesnake (*C. viridis viridis*), Tancitaran dusky rattlesnake (*C. pusillus*), Northern Mojave rattlesnake (*C. scutulatus scutulatus* type B) and Northern Pacific rattlesnake (*C. v. oreganus*)) had one single band in the five-step western blot. DV-2LD#2 did not bind to the non-hemorrhagic venoms and reacted with 50% of the hemorrhagic venoms used in this study. The monoclonal antibody, CAH, reacted with all the hemor-

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rhagic venoms except for the venom of the King cobra (*Ophiophagus hannah*) and did not react with the non-hemorrhagic venoms. The hemorrhagic binding site of CAH monoclonal antibody and the antihemorrhagin in Virginia opossum are different binding sites. The five-step western blot will be a very useful assay for determining hemorrhagic activity without using live animals.

INTRODUCTION

Venomous snakes have an effective way of capturing and digesting prey. Snake venom is a complex mixture of many toxins which allows them to immobilize their prey without a chase or struggle. Yet, certain prey species and most snakes have a natural resistance to venoms and can escape death. The natural resistance of snakes to their own venoms is documented in the literature (Deoras and Mhasalkar, 1963; Clark and Voris, 1969; Straight *et al.*, 1976).

Virginia opossums (*Didelphis virginiana*) are known to tolerate crotaline venom without developing hemorrhage, or any other side effects normally associated with hemorrhagic venoms. The Virginia opossum has a natural resistance to induced snakebites and massive intravenous injection of venom (Kilmon, 1976; Werner and Vick, 1977; Pérez *et al.*, 1978; Perales *et al.*, 1986). Menchaca and Pérez (1981) isolated an antihemorrhagic protein from the serum of *D. virginiana*. The purified factor was homogeneous by polyacrylamide disc electrophoresis, had a p*I* of 4.1, and a molecular weight of 68 000 Da. Rodríguez-Acosta *et al.* (1995) reported that a common opossum (*D. marsupialis*) serum fraction neutralized hemorrhagic and proteolytic fractions of Fer-de-lance (*Bothrops lanceolatus*) venom.

Catanese and Kress (1992) reported isolation of an inhibitor from Virginia opossum, oprin (opossum proteinase inhibitor), which inhibited *C. atrox* hemorrhagin HT-b and showed similar inhibition of hemorrhagic activity of *C. atrox* α-, β- and γ-proteinase. However, oprin had no antihemorrhagic activity against *C. atrox* HT-a, the most hemorrhagic protein isolated from *C. atrox*, which still produces hemorrhage after a 30-fold excess of oprin. Catanese and Kress (1993) reported another inhibitor from Virginia opossum serum, α_1-proteinase inhibitor (α_1-PI), with a molecular weight similar to oprin, but with a different isoelectric pH, which inhibited *C. atrox* HT-a and HT-b. Serine protease inhibitors (serpins) from Virginia opossum serum (α_1-PI) were isolated by Kress (1986) which retained 100% activity when incubated with *C. atrox* α-proteinase and had 96% activity when incubated with Eastern diamondback rattlesnake (*C. adamanteus*) proteinase II. Similar serpin analogs found in human plasma had no activity and 5% activity when incubated with these same venom proteinases, respectively.

The neutralization of hemorrhage is achieved by the binding of antihemorrhagins in Virginia opossum to hemorrhagins in snake venom. These antihemorrhagic factors are not antibodies since they have different physical properties and do not show proteolytic activity (Menchaca and Pérez, 1981; García and Pérez, 1984). Binding of antihemorrhagins from *C. atrox* serum to the HT-e on a polyacrylamide gel was shown by Weissenberg *et al.* (1991), Takeya *et al.* (1989) and Catanese and Kress (1992). Tanizaki *et al.* (1991) showed binding of a proteinase inhibitor from the plasma of Jararaca (*Bothrops jararaca*) to a J protease, a metalloproteinase in the venom, by gel filtration chromatography.

In this paper, we reported an *in vitro* assay which can specifically detect hemorrhagins in snake venoms in a modified western blot. This assay suggests binding of antihemorrhagin(s) found in Virginia opossum serum to hemorrhagins in snake venoms. The complex (hemorrhagin/antihemorrhagin) is detected by DV-2LD#2 antibody which is specific for Virginia opossum serum. With the development of other monoclonal antibodies for antihemorrhagins, hemorrhagic venoms can be determined without using live animals by the five-step western blot.

MATERIALS AND METHODS

Venoms/snakes

C. atrox venom was collected from Big Springs, TX, U.S.A. and lyophilized. Blacktail rattlesnake (*C. molossus molossus*), Great Basin rattlesnake (*C. v. lutosus*), Northern Mojave rattlesnake (*C. s. scutulatus* type A and *C. s. scutulatus* type B) venoms were donated by the Veterans Affairs Hospital at Salt Lake City, UT, U.S.A. The Olive-brown sea snake (*Aipysurus leavis*), Puff adder (*Bitis arietans*), King cobra (*O. hannah*), and Chinese cobra (*N. naja atra*), Tancitaran dusky rattlesnake (*C. pusillus*), and Rock rattlesnake (*C. lepidus*) were donated by Sherman A. Minton. *C. v. viridis*, *C. v. oreganus*, Broad-banded copperhead (*Agkistrodon contortrix laticinctus*), and Western cottonmouth (*A. piscivorus leucostoma*) venoms were obtained from individual specimens housed in the serpentarium at Texas A & M University-Kingsville, Kingsville, TX, U.S.A. Since many of the snake venoms were gifts, it is not known how many snakes were used. Lyophilized venoms were suspended in Milli-Q water at a concentration of 5 mg/ml. The venoms were centrifuged using a Beckman Avanti™ 30 Centrifuge at 5911 × g for 10 min and filtered using a Millipore MillexHV 0.45 μm filter unit. The venom samples were stored at −90°C.

Serum collection

Virginia opossums were collected within the Kingsville, TX city limits by the Kleberg County Animal Control officials. Virginia opossums were anesthetized with isofluorane and bled by heart puncture using a 15 gauge needle. Blood was allowed to coagulate in 50 ml Fisherbrand centrifuge tubes, for 24 h at 4°C. Serum was separated from erythrocytes by centrifugation in a Beckman Avanti™ 30 centrifuge, at 5911 × g for 15 min. Serum samples were stored at −90°C. Gray woodrats were collected at Texas A & M University-Kingsville Biological Station at Site 55, Kleberg County, TX, U.S.A. The same procedure that was used to obtain Virginia opossum sera was employed to collect gray woodrat sera.

Hemorrhagic and antihemorrhagic assays

Hemorrhagic and antihemorrhagic activities were measured in the back of New Zealand white rabbits (*Oryctolagus cuniculus*) by the same procedure used by Soto *et al.* (1988). The minimal hemorrhagic dose (MHD) is defined as the amount of venom required to cause 10 mm hemorrhagic diameter in the back of a rabbit after 15 h.

Monoclonal antibodies

Monoclonal antibodies were produced by a method reported by Pérez *et al.* (1984). The monoclonal antibodies (DV-2LD#2 and CAH) were produced from partially purified antihemorrhagic proteins from *D. virginiana* serum and hemorrhagic proteins from *C. atrox* venom, respectively. After immunization of BALB/c mice, the spleen cells were fused with SP2/0 cells, and the positive cell lines were cloned by limiting dilutions.

DV-2LD#2 blocking activity

Equal volumes of Virginia opossum serum (1/32) were mixed with crude DV-2LD#2 crude ascites fluid and reacted at 25°C for 1 h. Equal volumes of this mixture were reacted with MHD (50 μg/ml) of *C. atrox* venom and incubated for 1 h at 25°C. One-hundred microliters were injected intracutaneously into the back of a New Zealand white rabbit. The rabbit was sacrificed 15 h later and hemorrhagic areas were measured.

Native-PAGE electrophoresis

Two and one-half micrograms of each venom were placed on 8–25% polyacrylamide gradient gels. The venoms and Virginia opossum serum were electrophoresed as described previously (Rael *et al.*, 1984). The proteins were visualized using silver nitrate stain.

Western blot and five-step western blot

After electrophoresis, the proteins were transferred to an Immobilon®-P membrane using a trans-blot dish containing 0.7% acetic acid as described previously (Anaya *et al.*, 1992). The gel and membrane were soaked in Milli-Q water and separated carefully with forceps. The membrane was incubated in 5% powdered milk (Rael *et al.*, 1993) in 0.01 M phosphate buffered saline (PBS), pH 7.0. The Immobilon membrane was incu-

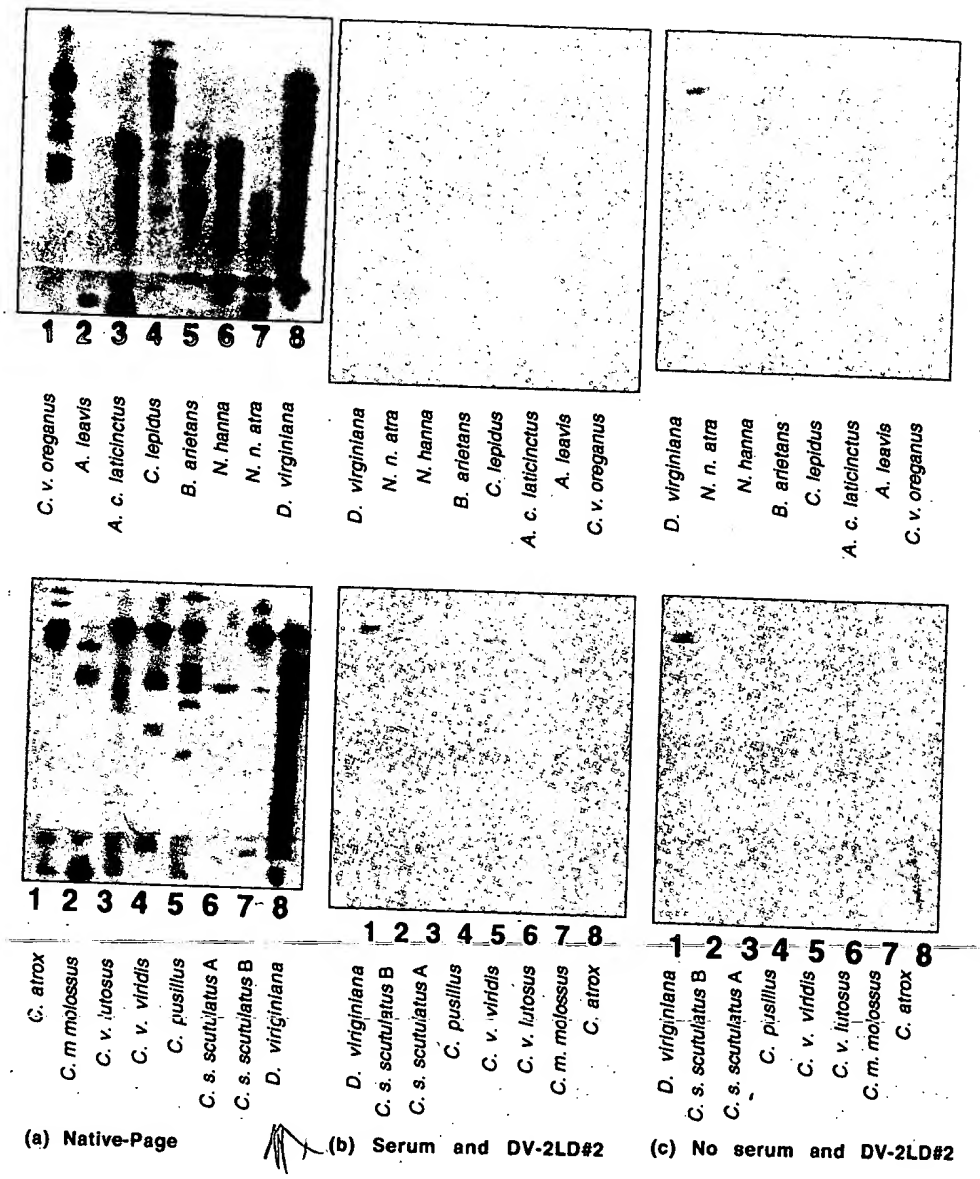
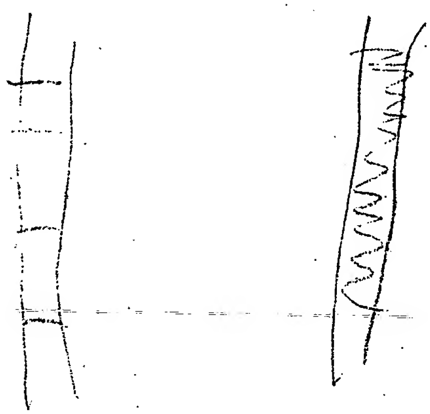


Fig. 1. Native-PAGE electrophoresis and a five-step western blot of 14 different venoms using *D. virginiana* serum and DV-2LD#2 monoclonal antibody. (a) Native-PAGE gels of *D. virginiana* serum and 14 different snake venoms. Two and one-half micrograms of snake venoms were separated by electrophoresis. (b) Venom bands on the gel were transferred by diffusion onto a Millipore Immobilon membrane and a five-step western blot was performed using *D. virginiana* serum and DV-2LD#2 monoclonal antibody. Most of these venoms tested positive with DV-2LD#2 monoclonal antibody. (c) Venom bands from another set of native-PAGE gel were transferred onto a Millipore Immobilon. When no Virginia opossum was used, the 14 venoms did not react with DV-2LD#2 monoclonal antibody.

Venom finder

olfactory genes



+ Black & Pelt.

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bated with Virginia opossum serum (24 mg/ml) or gray woodrat serum for 2 h at 25°C. After washing with PBS, the membrane was placed in 10 ml of ascites fluid containing DV-2LD#2 (0.27 mg/ml) or CAH (0.2 mg/ml) monoclonal antibodies and incubated for 2 h at 25°C. The membrane was rinsed with PBS and then incubated with 10 ml of a 1:3000 dilution of BIO-RAD goat anti-mouse IgG conjugated with horseradish peroxidase for 2 h at 25°C. The proteins were developed with 4-chloro-1-naphtol and hydrogen peroxide (Rael *et al.*, 1993). Immediate results were obtained if there was a strong antibody–antigen reaction.

RESULTS

A DV-2LD#2 monoclonal antibody was produced against and reacted specifically with three proteins in Virginia opossum serum (Lane 1 in Fig. 1(b),(c)). DV-2LD#2 monoclonal antibodies reacted indirectly in a five-step western blot with six venoms (*C. atrox*, *C. v. lutosus*, *C. v. viridis*, *C. pusillus*, *C. s. scutulatus* type B and *C. v. oreganus* venoms) when *D. virginiana* serum was used (Fig. 1(b)). *C. v. oreganus* and *C. pusillus* venoms reacted to a lesser degree in the five-step western blot. The DV-2LD#2 monoclonal antibody did not react with the same venoms when the Virginia opossum serum was excluded (Fig. 1(c)). The six venoms in this study that gave positive results in the five-step western blot were hemorrhagic venoms from the genus *Crotalus*. Three hemorrhagic venoms, *C. m. molossus* and *C. lepidus* from the genus *Crotalus*, did not react in the five-step western blot. The hemorrhagic Elapidae venom, *O. hannah*, did not react in the five-step western blot. Three of the venoms in this study were not hemorrhagic (*A. leavis*, *C. s. scutulatus* A, and *N. n. atra*) and did not react in the five-step western blot. The five-step western blot was used to distinguish between *C. s. scutulatus* type B venom (hemorrhagic) and *C. s. scutulatus* type A venom (non-hemorrhagic). The two *Agkistrodon* venoms tested, which are hemorrhagic, did not react in the five-step western blot. The immunoblot for *A. p. leucostoma* was not shown. The Viperidae venom, *B. arietans*, was also negative in the five-step western blot.

Table 1. Five-step western blot summary of 15 venoms using *D. virginiana* serum and two monoclonal antibodies (DV-2LD#2 and CAH)

Venom type	MHD (μg)*	Antihem. titer (serum)†	Serum + DV-2LD#2‡	Serum + CAH	CAH§
A. leavis¶	–	–	no	no	no
A. c. laticinctus	4.2	128	no	yes	yes
A. p. leucostoma‖	10.0	256	no	yes	yes
B. arietans¶	1.5	2	no	yes	yes
C. atrox	2.5	128	yes	yes	yes
C. lepidus	6.0	128	no	yes	yes
*C. m. molossus***	5.0	256	no	yes	yes
C. pusillus	2.0	128	yes	yes	yes
*C. s. scutulatus A***	–	–	no	no	no
*C. s. scutulatus B***	8.0	128	yes	yes	yes
C. v. lutosus	2.1	128	yes	yes	yes
C. v. oreganus	4.0	16	yes	yes	yes
C. v. viridis	1.5	16	yes	yes	yes
O. hannah¶	25.0	††	no	no	no
N. n. atra¶	–	–	no	no	no

* The MHD is defined as the amount of venom resulting in a 10 mm hemorrhagic area.
† The antihemorrhagic titer is defined as the reciprocal of the highest dilution of *D. virginiana*.
‡ DV-2LD#2 is a monoclonal produced against purified antihemorrhagins in Virginia opossum serum.
§ CAH is a monoclonal antibody produced against a hemorrhagic fraction of *C. atrox* venom.
¶ Venoms donated by Sherman A. Minton.
‖ The immunoblot for *A. p. leucostoma* was not shown.
** Venoms donated by James Glenn, VA Hospital, Salt Lake City, UT.
†† Soto *et al.* (1988).

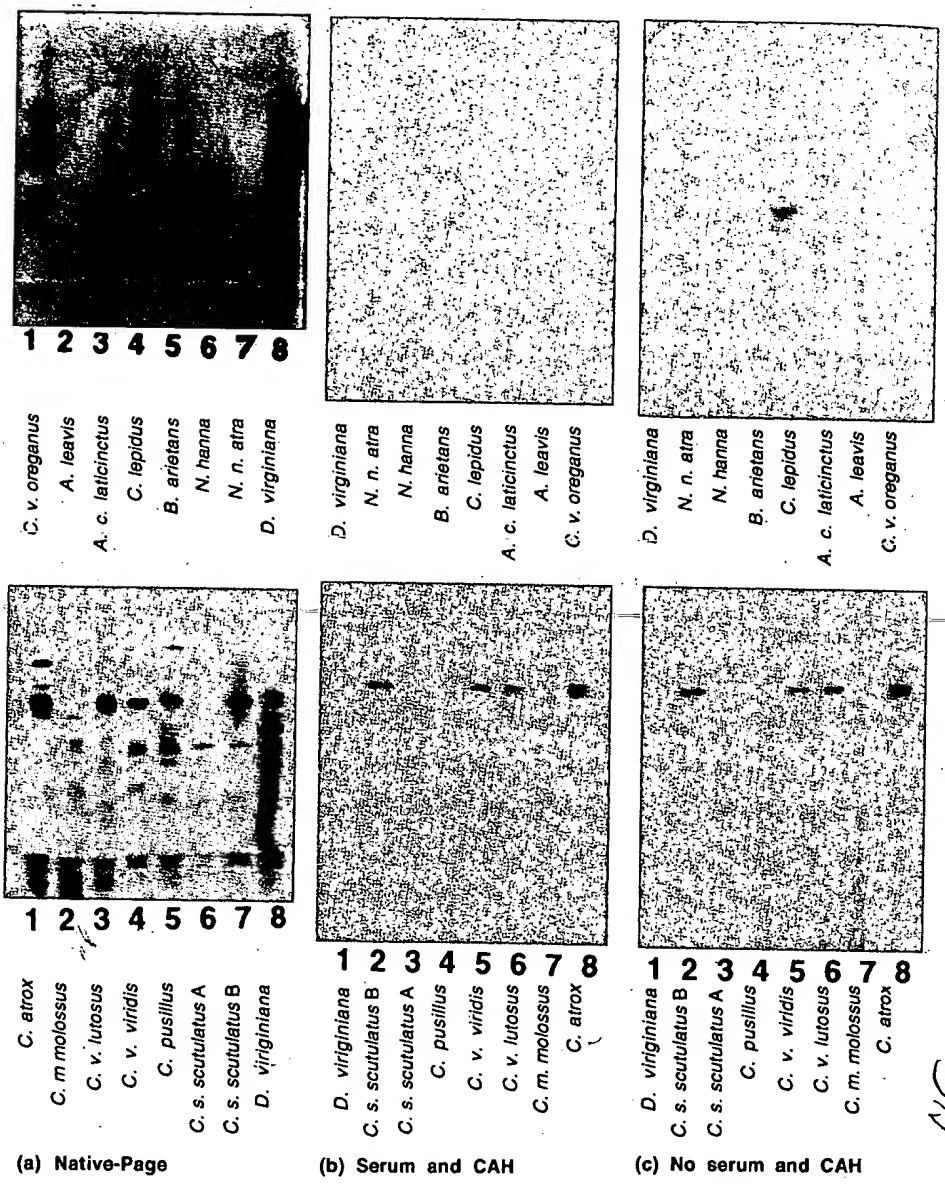

Fig. 2. Native-PAGE electrophoresis and a five-step western blot profiles of *D. virginiana* serum and 14 different venoms using CAH monoclonal antibody. (a) Native-PAGE gels of *D. virginiana* serum and 14 different snake venoms. Two and one-half micrograms of snake venom were separated by electrophoresis using a Pharmacia PhastSystem and stained with silver nitrate. (b) Protein bands on the native-PAGE gels were transferred by diffusion onto a Millipore Immobilon membrane and a five-step western blot was performed using opossum serum and CAH monoclonal antibody. (c) Protein bands from another set of native-PAGE gel were transferred onto a Millipore Immobilon and a western blot was performed using CAH monoclonal antibody (no opossum serum was used). Virginia opossum did not block CAH monoclonal antibody since the pattern in (b) and (c) were identical

Two of the most hemorrhagic venoms were *C. v. viridis* and *B. arientans* (1.5 MHD) and the least hemorrhagic venoms was *O. hannah* (25 MHD). All the hemorrhagic venoms were neutralized by *D. virginiana* serum. Virginia opossum serum neutralized *B. arietans* venom at a very high serum concentration (Table 1).

CAH monoclonal antibody was originally produced for hemorrhagic fractions of *C. atrox* venom and reacted with 11 hemorrhagic venoms tested, with the exception of *O. hannah* venom (Table 1). The three non-hemorrhagic venoms (*C. s. scutulatus* type A, *A. leavis*, and *N. naja atra)* gave negative western blots when CAH monoclonal antibody was used. Snake venoms with and without Viriginia opossum serum showed similar blotting patterns. The antihemorrhagins in Viriginia opossum serum did not prevent CAH from binding to the 11 venoms (*C. s. scutulatus* type B, *C. v. viridis*, *C. v. lutosus*, *C. atrox*, *C. pusillus*, *C. m. molossus*, *C. v. oreganus*, *A. c. laticinctus*, *A. p. leucostoma*, *C. lepidus and B. arietans*). CAH reacted most strongly with *C. s. scutulatus* type B, *C. v. viridis*, *C. v. lutosus*, *C. atrox*, *C. v. oreganus*, and *B. arietans* venoms (Fig. 2(b),(c)).

DISCUSSION

Many investigators have reported that antihemorrhagic factors in Virginia opossum serum neutralize hemorrhagins in many snake venoms (Pérez *et al.*, 1979; Tanizaki *et al.*, 1991; Weissenberg *et al.*, 1991). It has been reported that hemorrhagins are neutralized by binding to antihemorrhagins in Virginia opossum serum (Takeya *et al.*, 1989; Weissenberg *et al.*, 1991; Tanizaki *et al.*, 1991; Catanese and Kress, 1992). Since the mechanism of neutralization is through binding, then the antihemorrhagin(s) in Virginia opossum serum could be used to develop a five-step western blot.

In this study, a five-step western blot was developed in which the antihemorrhagin(s) in Virginia opossum serum bind to hemorrhagins in snake venoms. The proposed mechanism of binding is outlined in Fig. 3. Virginia opossum serum and DV-2LD#2 monoclonal antibody reacted with hemorrhagins in venoms after electrophoresis. The separated hemorrhagins reacted nonspecifically with *D. virginiana* serum which provided

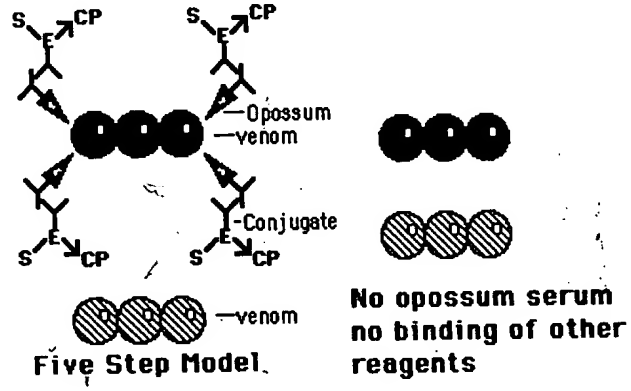

Fig. 3. Five-step western blot model. The spheres represent venom proteins separated by electrophoresis. The wedge represent antihemorrhagic molecules in Virginia opossum serum which links the DV-2LD#2 monoclonal antibody to hemorrhagins in venom. The black "Y" represents the DV-2LD#2 monoclonal antibody and the black "Y" with the "E" represents the conjugate. The enzyme converts the substrate "S" to colored product "CP". See Fig. 2 for actual five-step western blot

the linkage to DV-2LD#2 monoclonal antibody. The antihemorrhagic activity in Virginia opossum serum was blocked with DV-2LD#2 monoclonal antibody. This suggested that the DV-2LD#2 monoclonal antibody was specific for antihemorrhagins. DV-2LD#2 monoclonal antibody did not recognize the hemorrhagic venoms when the serum from *N. micropus* was used. There appears to be no cross reaction between DV-2LD#2 monoclonal antibody and antihemorrhagins in *N. micropus* serum.

In a five-step western blot there were two bands present with the *C. atrox* venom and one band with the other venoms (Fig. 1(b)). The location of the bands in the five-step western blot were identical and were hairline (very thin) bands. This suggests that the proteins are similar since their electrophoretic mobility and binding characteristics were identical. The hemorrhagic venoms from *C. pusillus* and *C. v. oreganus* had a weak response to Virginia opossum serum and DV-2LD#2 monoclonal antibody.

The DV-2LD#2 monoclonal antibody did not react with four of the Crotalidae venoms: *C. m. molossus*; *C. lepidus*; *A. c. laticinctus*; and *A. p. leucostoma*. The hemorrhagins in these venoms may be reacting with the different antihemorrhagins in Virginia opossum serum which did not bind to DV-2LD#2 monoclonal antibody. This supports the finding of Catanese and Kress (1992) that more than one antihemorrhagin is found in Virginia opossum serum. Other monoclonal antibodies need to be found which recognize hemorrhagins in *C. m. molossus*; *C. lepidus*; *A. c. laticinctus*; *A. p. leucostoma* and *O. hannah* venoms. Virginia opossum serum neutralized all hemorrhagic venoms tested in this study but the neutralization was not equal. Different serum concentrations were required to neutralize various venoms. The MHD for *B. arietans* was 1.5 μg and caused a hemorrhagic area 10 mm in diameter. *B. arietans* had one of the highest MHD and required more Virginia opossum serum for neutralization of hemorrhagic activity. Some venoms are more difficult to neutralize than others (Table 1). Soto *et al.* (1988) reported the serum of *D. virginiana* neutralized all hemorrhagic venoms tested from three different families.

Since there are seven hemorrhagins found in *C. atrox* venom, one would expect multiple bands for *C. atrox* in the five-step western blot. Possible reasons for not seeing multiple bands in the five-step western blot are: (1) snake venom from a single source may only have one or two hemorrhagins; however, this is not the case with *C. atrox* venom, which was a pooled venom sample; (2) the multiple hemorrhagins may have identical electrophorectic mobility at the pH used, and all hemorrhagins migrate as a single band; (3) the concentration of the minor hemorrhagic components of venoms or antihemorrhagins from Virginia opossum may be low and are not showing up in the five-step western blot; or (4) there could be multiple antihemorrhagins found in Virginia opossum serum and not all are recognized by DV-2LD#2 monoclonal antibody. Catanese and Kress (1993) reported more than one factor in Virginia opossum which reacted with venom.

CAH monoclonal antibody was produced in response to *C. atrox* venom and reacted with all hemorrhagic venoms, except for *O. hannah* venom, in a western blot; however, CAH did not neutralize hemorrhagic activity in the snake venoms tested. CAH monoclonal antibody binds to hemorrhagic molecules but the binding site is different than the active site recognized by antihemorrhagins in Virginia opossum serum. In a western blot the antihemorrhagin found in Virginia opossum serum did not compete for the same active site as the CAH monoclonal antibody (Fig. 2). This strongly suggested that antihemorrhagins and CAH are not competing for same sites.

DV-2LD#2 monoclonal antibody indirectly recognized 50% of the hemorrhagic venoms tested; and no non-hemorrhagic venoms were recognized. It is clearly under-

stood that the production of other monoclonal antibodies specific for antihemorrhagins is necessary for the detection of hemorrhagic venoms that were negative in this study. Venoms from different geographical locations need to be tested. The five-step western blot will be important in screening snake venoms & animal sera in phylogenetic studies, following purification of hemorrhagins and antihemorrhagins, screening for recombinant products in cloning experiments, and studying the mechanism of neutralization without using live animals.

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